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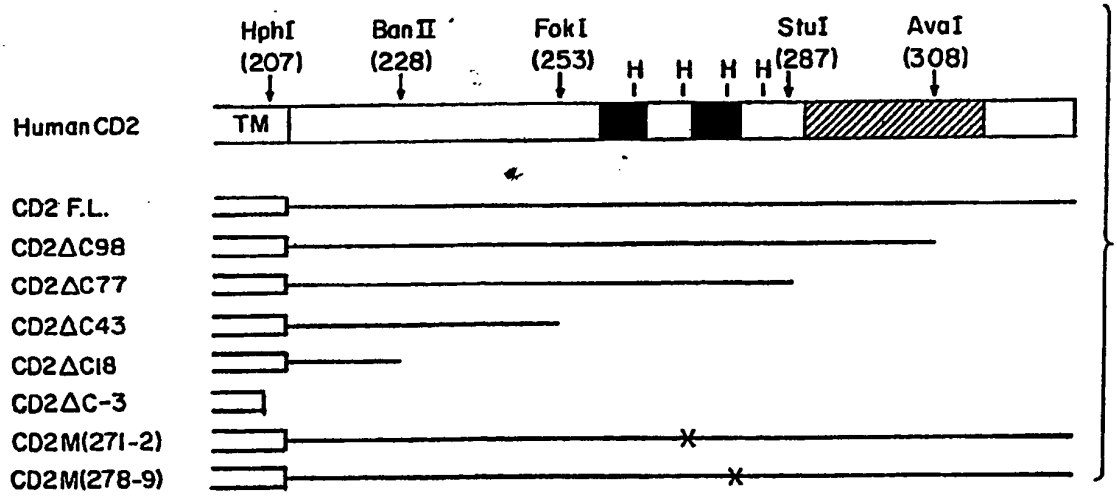


(57) Abstract

This invention pertains to soluble peptides and DNA encoding the peptides that correspond to the cytoplasmic domain of human CD2 glycoprotein or portion thereof which is necessary for transduction of a CD2-mediated T lymphocyte activation signal into a cell. The invention also pertains to cells transformed to express a CD2 surface receptor, and to methods for screening substances which stimulate or block CD2 receptor function.

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# SOLUBLE CYTOPLASMIC DOMAIN OF HUMAN CD2 RECEPTOR

## Background

The 50 KD CD2 (T11) surface glycoprotein, originally defined as the sheep erythrocyte receptor, plays an important role in T lymphocyte (T cell) activation as well as in facilitating adhesion between T lymphocytes and their cognate partners. Three distinct epitopes on the 50 KD CD2 molecule have been identified. The $T11_1$ and $T11_2$ epitopes are expressed on resting as well as activated T cells. Anti-$T11_3$ antibodies recognize a spatially distinct epitope that is preferentially expressed on mitogen- or antigen-activated lymphocytes.

Perturbation of the extracellular domain of CD2 by its ligand, lymphocyte function-associated antigen 3 (LFA-3), or certain anti-CD2 monoclonal antibodies (i.e., mitogenic combination of anti-$T11_2$ and anti-$T11_3$) provides signals that synergize to augment T cell antigen receptor-mediated stimulation. Following such membrane perturbation, a rapid turnover in polyphosphoinositides occurs, accompanied by an increase in cytosolic calcium concentration. Subsequently, nuclear activation follows including IL-2

gene expression resulting in lymphokine secretion and proliferation.

The primary structure of human CD2 by protein microsequencing and cDNA clones has been elucidated (Sayre et al., Proc. Natl. Acad. Sci. USA 84:2941-2945 (1987); Sewell et al., Proc. Natl. Acad. Sci. USA 83:8718-8722 (1986); Seed et al., Proc. Natl. Acad. Sci. USA 84:3365-3369 (1987)). The protein predicted by cDNA sequence is comprised of the following three domains: (i) a hydrophilic 185 amino acid extracellular domain accounting for more than half of the molecule and bearing only limited homology to members of the immunoglobulin supergene family including T4, the T cell surface glycoprotein; (ii) a single characteristic hydrophobic 25 amino acid transmembrane domain; and (iii) a 117 amino acid cytoplasmic domain, rich in proline and basic amino acid residues.

Additionally, cDNAs encoding the murine and rat equivalent of CD2 have been cloned and sequenced to show >50% amino acid sequence identity (Clayton et al., Eur. J. Immunol. 17:1367-1370 (1987); Sewell et al., Eur. J. Immunol. 17:1015-1020 (1987); Williams et al., J. Exp. Med. 165:368-380 (1987); Clayton et al., J. Immunol. 140:3617-3621 (1988); Diamond et al., Proc. Natl. Acad. Sci. USA 85:1615-1619 (1988); Lang et al., EMBO J. 7:1675-1682 (1988)).

The highest degree of homology between human and murine species is found in the cytoplasmic domain (59% at the amino acid level). In both species, the cytoplasmic domain is unique with respect to its multiple proline (21%) and basic residues and by

secondary structural predictions, suggesting that it has an extended non-globular conformation.

Summary of the Invention

This invention pertains to soluble peptides which correspond to the cytoplasmic domain of human CD2 glycoprotein, or a portion of the cytoplasmic domain, which is necessary for transduction of a CD2-mediated T lymphocyte activation signal into a cell. In particular, the soluble peptides can comprise the entire 117-residue amino acid sequence of the mature human CD2 cytoplasmic domain or 77 amino acid residues corresponding to the amino-terminus of the CD2 cytoplasmic domain. Preferred peptides include a 35 amino acid sequence corresponding to a portion of the cytoplasmic domain which is necessary for transduction of a CD2-mediated T lymphocyte activation signal into a cell.

The invention also pertains to an isolated DNA sequence encoding the cytoplasmic domain of human CD2 glycoprotein or a portion thereof.

This invention further pertains to cells which normally do not bear CD2 receptors, which have been transformed by an expression vector containing a CD2-encoding DNA, to express CD2 protein on their surface. The transformed cells can be used to screen for substances which are agonistic or antagonistic to CD2-mediated cellular responses.

The invention also pertains to chimeric cell surface receptors containing a CD2-derived cytoplasmic domain and a non-CD2 extracellular domain. The chimeric receptors are encoded by chimeric gene

contructs comprising DNA encoding the extracellular domain of a non-CD2 cell surface receptor linked to DNA encoding the cytoplasmic domain of human CD2 glycoprotein or portion thereof which is necessary for transduction of a CD2-mediated T lymphocyte activation signal into a cell. Cells transformed with the chimeric gene can express the chimeric surface receptor and can be used to screen for substances which are agonistic or antagonistic to the non-CD2 receptor-mediated response.

Brief Description of the Drawings

Figure 1 is the DNA sequence of the human CD2 cytoplasmic domain.

Figure 2 is a comparison of predicted transmembrane and cytoplasmic domain sequences of human and mouse CD2. Amino acid residues are designated in single letter code with the transmembrane regions underlined. Identical residues in human and mouse CD2 sequences are starred.

Figure 3 is a schematic representation of the transmembrane and cytoplasmic regions of human CD2 and variant molecules. Constructs of full length, deletion and substitution mutants of CD2 are diagrammed. The region most conserved between human and mouse CD2 is stippled, and the two repeating PPPGHR segments are marked in black. The X denotes the histidine residues thought to form a putative binding site. The restriction sites that generate the truncated CD2 molecules are marked by arrows with numbers in parenthesis corresponding to amino acid residues.

Figure 4 is a schematic representation of the structure of the DOL retroviral expression vector.

Figures 5a-f are graphic representations of flow cytometric analysis of CD2 expression on murine T cells. Indirect immunofluorescence assays were carried out using the anti-$T11_1$ monoclonal antibody (3T4-8B5) (thick line) and compared to an irrelevant antibody (1HT4-4E5) (thin line) as background.

Figures 6a and 6b are audioradiograms for immunoprecipitation of CD2 from lysates of iodinated cell lines.

Figures 7a-f are histograms of various T cell lines for the measurement of cytosolic $Ca^{+2}$ by indo-1 fluorescence.

Figures 8a-d represent indirect immunofluorescence detection of murine CD3 and human CD2 on the surface of modulated and unmodulated CD2 FL.

Figure 8e represents the measurement of $[Ca^{+2}]_i$ vs. time in modulated CD2 FL cells after stimulation with anti-$T11_2$ and anti-$T11_3$ antibodies (black arrow) or calcium ionophore (open arrow).

Figure 9 is a graphic representation of ($^3H$) thymidine incorporation of stimulated or unactivated murine cells, CD2 FL or 3DO54.8, which were grown on IL2-dependent murine CTLL-20 cells. Cells were stimulated with anti-$T11_2$ and anti-$T11_3$ antibodies.

Figures 10a and 10b are histogram representations of IL-2 production by CD2 FL, CD2Δ98, CD2Δ77, CD2Δ43, CD2Δ18, 3DO54.8 and murine T cell line CD2 M271-2 upon antigen (ovalbumin) or anti-$T11_2$ and anti-$T11_3$ stimulation.

Detailed Description of the Invention

The present invention pertains to soluble peptides having an amino acid sequence that corresponds to the cytoplasmic domain of human CD2 glycoprotein or a portion thereof, which is necessary for transduction of a CD2-mediated T lymphocyte activation signal into a cell. The peptides of this invention are soluble in aqueous medium. In general, the soluble peptides include an amino acid sequence of approximately 35 amino acid residues corresponding to a portion of the cytoplasmic domain of CD2 necessary for transduction of a CD2-mediated T cell activation signal into a cell. This region contains four histines at amino acid position 264, 271, 278 and 282 and includes two tandomly repeated segments (PPPGHR, amino acids 260-265 and 274-279). Mutations at positions 278-279 alter the structure of the second repeat without affecting cell activation, suggesting that the more carboxy-terminal repeat is not required for cell activation. The peptide has the following 35 amino acid residues.

253 Ala Thr Ser Gln His Pro Pro Pro Pro Pro
263 Gly His Arg Ser Gln Ala Pro Ser His Arg
273 Pro Pro Pro Pro Gly His Arg Val Gln His
283 Gln Pro Gln Lys Arg

In one embodiment, the soluble peptides have an amino acid sequence that correspond to the entire amino acid sequence of the mature human CD2 cytoplasmic domain and have the following 117 amino acid residue sequence:

211 Thr Lys Arg Lys Lys Gln Arg Ser Arg Arg
221 Asn Asp Glu Glu Leu Glu Thr Arg Ala His
231 Arg Val Ala Thr Glu Glu Arg Gly Arg Lys
241 Pro His Gln Ile Pro Ala Ser Thr Pro Gln
251 Asn Pro Ala Thr Ser Gln His Pro Pro Pro
261 Pro Pro Gly His Arg Ser Gln Ala Pro Ser
271 His Arg Pro Pro Pro Pro Gly His Arg Val
281 Gln His Gln Pro Gln Lys Arg Pro Pro Ala
291 Pro Ser Gly Thr Gln Val His Gln Gln Lys
301 Gly Pro Pro Leu Pro Arg Pro Arg Val Gln
311 Pro Lys Pro Pro His Gly Ala Ala Glu Asn
321 Ser Leu Ser Pro Ser Ser Asn

The soluble peptides of this invention, however, can be a functional portion of the cytoplasmic domain which is necessary for transduction of a CD2-mediated T cell activation signal into a cell. Preferably, the soluble peptide has about 77 amino acid residues corresponding to the amino-terminal portion of the CD2 cytoplasmic domain. Additional amino acid residues can be attached to the carboxy-terminus of the 77 amino acid sequence. Specifically, 21 amino acid residues corresponding to the carboxy-terminus of the CD2 cytoplasmic domain (i.e., amino acid position 288-308) can be attached to the soluble peptide of 77 amino acid residues. The resulting 98 residue peptide is functionally capable of transducing a CD2-mediated activation signal into a cell.

The soluble CD2 peptides of this invention include analogous or homologous sequences which encode proteins capable of transducing a CD2-mediated activation signal

into a cell. These peptides can include sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include glycine, alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic and glutamic acid.

In addition, the peptide structure can be modified by deletions, additions, inversion, insertions or substitutions of one or more amino acid residues in the sequence which do not substantially detract from the functional properties of the peptide. Naturally occurring allelic variations and modifications are included within the scope of the invention so long as the variation does not substantially reduce the ability of the peptide to transduce a CD2-mediated T cell activation signal into a cell.

Soluble peptides of this invention can be made by enzymatic fragmentation of human CD2 glycoprotein or a portion thereof, by peptide synthesis or recombinant

DNA technology. The peptides or modified equivalents thereof can be synthesized directly by standard solid or liquid phase chemistries for peptide synthesis. For example, the above amino acid sequence or modified equivalent thereof encoding the cytoplasmic domain of CD2 can be synthesized by the solid phase procedure of Merrifield.

Preferably, the soluble CD2 peptides will be produced by inserting DNA encoding the peptide (i.e., CD2 DNA which encodes the desired amino acid sequence of the cytoplasmic domain of CD2) into an appropriate vector/host system where it is expressed. The DNA sequence which encodes the cytoplasmic domain of native human CD2 is shown in Figure 1. The entire DNA sequence of CD2 has been described in U.S. Application Serial No. 06/932,871, filed November 18, 1986, by Reinherz et al. This native sequence or a variant thereof can be used.. For example, any of the possible DNA sequences which encode the amino acid sequences given above can be used. A substantial coding equivalent is any nucleic acid sequence which encodes an amino acid sequence having essentially the same functional characteristics as the native human CD2 cytoplasmic domain. In addition, the native DNA sequence of CD2 can be modified by deletion, insertion or substitution of nucleotides to yield peptides which exhibit substantially the same properties of the above-described soluble peptides. The DNA sequences can be chemically synthesized or they can be obtained from natural sources using recombinant DNA technology.

A variety of host-vector systems can be used to express the peptides of this invention. Primarily, the vector system must be compatible with the host cell used. Host-vector systems include, but are not limited to, the following: bacteria transformed with bacteriophage DNA, plasmid DNA or cosmid DNA; micro-organisms, such as yeast containing yeast vectors; mammalian cell systems infection with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus).

Any of the standard recombinant methods for the insertion of DNA into an expression vector can be used. The recombinant DNA vector can be introduced into appropriate host cells (bacteria, virus, yeast, mammalian cells or the like) by transformation, transduction or transfection (depending upon the vector/host cell system) and cultured to express the peptides of this invention.

This invention also pertains to cells which have been transformed to express CD2 protein on its surface. The cells are derived from cells which do not normally express CD2 protein or do not express the CD2 protein at significant levels on its surface. Preferably, a continuous non-CD2 bearing cell line is transformed with an expression vector containing CD2-encoding DNA to thereby cause the cell line to express CD2 protein on its surface. Typically, the expression vector will contain the DNA sequence encoding the extracellular, transmembrane and cytoplasmic domains of human CD2 glycoprotein. DNA encoding the native CD2 glycoprotein and portions of the protein have been previously

described in U.S. Patent Application Serial No. 06/932,871, filed November 18, 1986, by Reinherz et al., the teachings of which are incorporated herein by reference. The cytoplasmic domain of CD2, however, need only be a portion of cytoplasmic domain which is necessary for transduction of a CD2-mediated T lymphocyte activation signal into a cell, as described above. Suitable expression vectors include the DOL retroviral vector, baculovirus transfer vectors, pAc373 and pCDVI. The DOL retroviral expression vector is preferred.

The transformed cells which express the CD2 surface receptor can be used to screen for substances which are agonistic or antagonistic to CD2-mediated cellular response, such as drugs, chemicals and receptor-specific antibodies. In a screening method for agonistic substances, the transformed cell is contacted with a substance to be tested under conditions (including physiological levels of calcium) which would permit the substance to complex with the CD2 surface protein. Stimulation of the CD2 receptor is determined by a change in intracellular calcium concentration. Thus, calcium concentration is measured within the cell before and after the cell is contacted with the substance.

This system can be used to screen for substances which can block the CD2 surface receptor and inhibit CD2-mediated cellular response. The transformed cell is contacted with a substance to be tested for blocking activity and with a CD2 activating agent (i.e., a compound known to mediate CD2 activation of the cell) under conditions which would permit CD2 stimulation by

the activating agent. Intracellular calcium concentration is then measured as indicative of CD2-mediated cellular response.

The cytoplasmic domain of the CD2 receptor can be exploited to provide screening assays for substances that interact with cell surface receptors other than CD2. The CD2 cytoplasmic domain can be linked to a non-CD2 extracellular domain to make a chimeric receptor. The chimeric receptor has the extracellular region of a receptor of choice, but the intracellular domain of the CD2 receptor which, thus, produces a CD2-type intracellular response (calcium mediated). The chimeric receptor is made by producing an expressible chimeric gene construct which can be used to transform an appropriate host cell. The chimeric gene construct comprises DNA encoding the extracellular domain of a non-CD2 cell surface receptor; DNA encoding the transmembrane domain of a cell surface receptor (either CD2 or non-CD2); and DNA encoding the cytoplasmic domain of human CD2 glycoprotein or any portion of the domain which functions for transduction of a CD2-mediated T lympocyte activation signal into a cell. DNA encoding the transmembrane domain can correspond to DNA encoding the transmembrane domain of any cell surface receptor.

A cell (preferably one which does not ordinarily express the receptor of choice, such as mammalian cells of various tissue origins) is transformed with the chimeric gene construct and caused to express the chimeric surface receptor. These transformed cells can be used to screen for substances which are agonistic or

antagonistic to the receptor-mediated cellular response in screening assays, as described above. The CD2 cytoplasmic domain permits interaction with the receptors to be detected by the intracellular CD2-type calcium response.

The invention is further illustrated by the following Exemplification.

## Exemplification

### Production and Analysis of Human CD2 Cytoplasmic Domain

In order to characterize the functional and structural relationship of the cytoplasmic domain of the human CD2 molecule, a series of deletion mutation of the CD2 cDNA were produced as shown in Figure 3 encoding 98, 77, 43, 18 and -3 amino acids out of the 117 predicted cytoplasmic CD2 amino acid residues.

To construct the truncated CD2 molecule, the human CD2 cDNA, PB2 (Sayre, P.H. et al., Proc. Natl. Acad. Sci. USA 84:2941-2945 (1987)), was digested with restriction enzyme HphI, BanII, FokI, StuI and AvaI, respectively, and blunted with T4 DNA polymerase. To regenerate the last amino acid, to introduce a termination codon, and to provide a BamHI restriction recognition site, the HphI and BanII digests were ligated to the linker 5'-CTAAGGATCCTTAG-3' while FokI, StuI and AvaI digests were ligated to the linker 5'-TAAGGATCCTTA-3'.

The substitution mutants were generated by oligonucleotide-directed in vitro mutagenesis as described by Taylor, J.W. et al., Nucl. Acids Res. 13: 8764-8785 (1985). The synthetic olig nucle tides utilized for

mutagenesis were 5'-CAGGCACCTAGTGATGAGCCCCGCCTCCT-3' for CD2 M271-2 which changes the wild-type sequence CATCGT (His-Arg) into GATGAG (Asp-Glu) and 5'-CCGCCTCCTGGAGATGAGGTTCAGCACCAG-3' for CD2 M278-9 which changes the wild-type sequence CACCGT (His-Arg) into GATGAG (Asp-Glu).

The full length cDNAs as well as modified cDNAs were inserted into the retrovirus expression vector DOL (kindly provided by Dr. Thomas Roberts, Dana Farber Cancer Institute) (Figure 4). Selected restriction sites on the vector shown are: B, BamHI; RI, EcoRI; RV, EcoRV; H3, HindIII. The vector contains two promoters: MLV-LTR to drive the expression of the CD2 cDNA and the SV40 promoter to express the neomycin resistance gene (Korman, A. et al., Proc. Natl. Acad. Sci. USA 84:2150-2154 (1987)). The BamHI fragment of the replicative form of M13 mutant DNAs was subcloned into expressing vector DOL.

The plasmids containing full length or modified CD2 cDNAs were isolated and sequenced around the modified region by double-stranded sequencing before transfection by $Ca^{2+}$ precipitation into Ψ-2, a helper-free retrovirus packaging cell line. The murine T cell hybridoma 3DO54.8 cell line specific for ovalbumin in the context of the H-2 ($I\text{-}A^{d}$) molecule (Haskins, K. et al., J. Exp. Med. 157:1149-1169 (1983)) and lacking the human CD2 glycoprotein was infected with the defective retroviruses. Both transiently expressed and permanent viral stocks were used to infect the hybridoma in the presence of 8 μg/ml polybrene (Aldrich Chemicals, Milwaukee, WI).

Procedures for the growth of Ψ-2 cells, transfection of cells, harvest of virus, and infection of

cells were performed as described by Cepko, C.L. et al., Cell 37:1053-1062 (1984). G418 resistant clones were selected in the presence of 0.4 mg/ml G418 (Genetian, Gibco, Grand Island,NY) forty-eight hours after infection and wells containing single colonies expanded. The G418 resistant clones were analyzed for surface CD2 expression using an indirect immunofluorescence assay with anti-$T11_1$ and anti-$T11_2$ antibodies and positive clones were further sorted on the Epics V cell sorter.

Multiple clones corresponding to each type of truncation and expressing clearly detectable levels of surface CD2 were selected for further characterization. Clones used in subsequent functional studies were designated based on the nature of their CD2 cDNA retroviral insert: CD2 FL (full length) resulted from retroviral infection with the full length CD2 cDNA while CD2ΔC98, CD2ΔC77, CD2ΔC43, CD2ΔC18 and CD2ΔC-3 resulted from infection with retroviruses containing the entire extracellular and transmembrane segment of CD2 but only 98 or fewer of the 117 cytoplasmic residues. Cells were maintained in RPMI 1640 (Gibco) medium supplemented with 10% heat-inactivated fetal calf serum (Flow Labs, McLean, VA), 50 μM 2-mercaptoethanol (2-ME), 1mM sodium pyruvate, 2 mM L-glutamic acid, 1% penicillin-streptomycin (Whittaker M.A. Bioproducts, Walkersville, MD) and 0.4 mg/ml G418.

Expression of Human CD2 Molecules on Murine T Cells

Flow cytometric analysis of CD2 expression on murine T cells was carried out by indirect immunofluorescence assays using anti-$T11_1$ monoclonal antibody (3T4-8B5) and compared t an irrelevant antibody

(1HT4-4E5) as background. Ascites were used at a 1:200 dilution and $10^6$ cells incubation for 30 minutes at 4°C. After washing in RPMI 1640 with 2% fetal calf serum (FCS), bound antibodies were detected with a 1:40 dilution of fluorescein coupled goat anti-mouse IgG as a second antibody (Meloy, Springfield, VA). 10,000 cells were analyzed per sample on an Epics V cell sorter.

A representative pattern of reactivity with the anti-CD2 monoclonal antibody is shown in Figures 5a-f. Histograms represent the number of cells (ordinate) vs. log 10 fluorescence intensity (abscissa). All of these transfectants express comparable levels of CD2 (~5-10,000 copies/cell) except CD2ΔC18 and CD2ΔC-3, which express on the order of 50% the copy number. The surface expression of CD2 on ΔC-3 was unstable and did not allow functional analysis. For each clone, similar reactivities to those obtained with anti-$T11_1$ were found using the anti-$T11_2$ antibody while none of the unactivated clones were stained by the anti-$T11_3$ antibody.

To prove that individual clonal recipients of the truncated CD2 cDNAs express appropriately sized CD2 protein, immunoprecipitation and SDS-PAGE analysis of the corresponding $^{125}$I-labeled surface CD2 molecules was carried out. Immunoprecipitates were obtained from solubilized murine T cells using a non-specific (NS) antibody (mouse anti-human CD8) or an anti-CD2 antibody directed against the $T11_1$ epitope and run under reducing conditions over an SDS 10% polyacrylamide gel (PAGE).

$10\text{-}20 \times 10^6$ cells were surface labeled with 1 mCi $^{125}I$ (IMS 30, Amersham, Arlington Heights, IL) for 15 minutes at room temperature using the lactoperoxidase method by Sayre, P.H. et al., Proc. Natl. Acad. Sci. USA 84:1941-2934 (1987). Cell lysates were prepared in RIPA buffer containing 0.15 NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM NaF, 1 mM phenyl methyl sulfonyl fluoride and protease inhibitors. Cell lysates were precleared once with a formalin-fixed Staphylococcus aureus suspension, then with Affigel protein A beads (Pharmacia, Piscataway, NJ) coupled to an irrelevant antibody (anti-CD8; 21 Thy2D3) before overnight incubation at 4°C with the anti-CD2 (3T4-8B5) antibody coupled to beads. As seen in Figure 6, no human CD2 was immunoprecipitated from the murine 3DO54.8 cell line.

Immunoprecipitates were extensively washed with RIPA buffer and separated on a 10% SDS-PAGE after treatment with 5% 2-ME. The gel was dried and the autoradiograph exposed for two weeks at -70°C with intensifying screens. A contaminant band of 70KD was regularly detected in the autoradiograms despite extensive preclearing, as previously reported by Haskins, K. et al., J. Exp. Med. 157:1149-1169 (1983).

A 53KD band was identified of clone CD2 FL. Furthermore, parallel analysis of CD2 protein expressed by CD2ΔC98, ΔC77, ΔC43 and ΔC18 revealed that the molecular weights of surface CD2 (51,47,43 and 40 KD, respectively) correlated well with the expected truncations (Figure 6). Thus, the truncated CD2 cDNAs in DOL directs protein synthesis of the variant CD2 forms on the surface of the murine 3DO54.8 cells.

Elevation in Intracellular Free Calcium Mediated by CD2 Stimulation

Given that pertubation of the external domain of the CD2 molecule with a combination of anti-$T11_2$ and anti-$T11_3$ results in a rapid rise in $[Ca^{2+}]_i$ linked to IL-2 gene induction (Meuer, S.C. et al., Cell 36:897-906 (1984); Alcover, A. et al., Proc. Natl. Acad. Sci. USA 83:2614-2618 (1986)), we examined whether such a mitogenic combination of monoclonal antibodies was effective in stimulation of CD2 FL as well CD2ΔC cell lines.

Cytosolic $Ca^{2+}$ concentrations were determined according to Grynkiewicz et al., J. Biol. Chem. 260:3440-3450 (1985). Briefly, $2x10^6$ cells were loaded for 45 minutes at 37°C with 2μg/ml of the acetylmethyl ester of indo-1 (Molecular Probes, Junction City, OR) in 200 μl of RPMI 1640 plus 2% FCS. Cells were diluted 10 fold prior to analysis on an Epics V cell sorter.

Figures 7a-f show analyses of alteration in $[Ca^{2+}]_i$ after stimulation with anti-$T11_2$ and anti-$T11_3$ in various cells lines as measured with a calcium sensitive dye indo-1 and flow cytometric analysis in real time. Black and open arrows correspond to addition of anti-$T11_2$ + anti-$T11_3$ antibodies and calcium ionophore A23187, respectively. Upon $Ca^{2+}$ binding, indo-1 exhibits changes in fluorescein emission wavelengths from 480 to 410 nm (Grynkiewicz, G. et al., J. Biol. Chem. 260:3440-3450 (1985)). The ratio of 410/480 nm indo-1 fluorescence was recorded vs. real time and expressed in arbitrary units. One arbitrary unit represents approximately 200 nM $[Ca^{2+}]_i$.

For each determination, the baseline was assayed by recording indo-1 loaded cells for 1 minute. Anti-$T11_2$ (1old24C1) and anti-$T11_3$ (1mono2A6) ascites were added at a 1:100 final dilution. The $Ca^{2+}$ ionophore A23187 (Sigma Chemical, St. Louis, MO) was added at a 1 μg/ml final concentration. A clear rise in $[Ca^{2+}]_i$ (approximately a 200 nM increment) was observed upon stimulation of CD2 FL, CD2ΔC98 and CD2ΔC77 cells. The calcium rise occurs within 2 minutes after adding the stimulating antibodies, most likely corresponding to the time required for expression of the $T11_3$ epitope after anti-$T11_2$ stimulation, a phenomena observed previously for human T lymphocytes (Meuer, S.C. et al., Cell 36:897-906 (1984)).

In contrast, CD2ΔC43 and CD2ΔC18 clones were not triggered by anti-$T11_2$ and anti-$T11_3$ antibodies. As expected, the non-transfected line 3DO54.8 was also not stimulated. Given that an immediate $[Ca^{2+}]_i$ rise was observed after addition of the $Ca^{2+}$ ionophore A23187 (1 μg/ml final concentration), it is clear that cells were loaded with the fluorescent dye.

These data establish that a significant rise in $[Ca^{2+}]_i$ can be induced through human CD2 structures expressed on the membrane of murine T cells even in the absence of the carboxy-terminal 40 amino acid residues of the CD2 cytoplasmic segment. The present results with human CD2 are consistent with an independent report by Williams et al. (He, Q. et al., Cell 54:979-984 (1988)) demonstrating a requirement for the cytoplasmic tail of rat CD2 to trigger a rise in $[Ca^{2+}]_i$ after transfection of rat CD2 into the human Jurkat T cell line.

Influence of CD3 modulation on induction of $(Ca^{2+})_i$ elevation triggered through the human CD2 molecule

Since human CD2 function in human T lymphocytes requires expression of the CD3-Ti $\alpha/\beta$ complex (Meuer, S.C. et al., Cell 36:897-906 (1984); Pantaleo, G. et al., J. Exp. Med. 166:619 (1987); Alcover, A. et al., EMBO J. 7:1973-1977 (1988)), we examined whether the function of a human CD2 molecule within a murine cell line was linked to murine CD3-Ti.

In order to modulate the murine CD3 molecule from the surface CD2 FL, cells were incubated overnight with the hamster anti-mouse CD3$\epsilon$ (1152C11) (20 $\mu$g/ml) at 37°C. Cells were washed twice and an aliquot was removed to evaluate the effect of modulation on murine CD3 and human CD2 expression using a standard indirect immunofluorescence assay (with the 115 2C11 and 3T4-8B5 monoclonal antibodies, respectively). Modulated cells were loaded with indo-1 and cytosolic $[Ca^{2+}]_i$ was followed upon various stimuli. As shown in Figure 8, modulation of the murine CD3 molecule by the 115 2C11 resulted in nearly complete loss of the CD3 molecule from the cell surface of CD2 FL after incubation for 16 hours at 37°C while CD2 expression was unaltered.

Figures 8a-d represent indirect immunofluorescence detection of murine CD3 and human CD2 molecule, respectively, on the surface of unmodulated CD2 FL (Figure 8b) and modulated CD2 FL (Figure 8d). The latter was incubated with the 115 2C11 monoclonal antibody overnight (Leo, O. et al., Proc. Natl. Acad. Sci. USA 84:1374-1378 (1987)). Figure 8e represents measurement of $[Ca^{2+}]_i$ vs. time in

modulated CD2 FL cells after stimulation with anti-$T11_2$ and anti-$T11_3$ antibodies (black arrow) or calcium ionophore (open arrow). Such anti-CD3 modulated CD2 FL cells were no longer stimulated by anti-$T11_2$ and anti-$T11_3$ antibodies to increase $[Ca^{2+}]_i$. By contrast, the incubation of CD2 FL cells under similar conditions with anti-human CD3 antibody (Leu4) did not have any effect. This result suggests that regulation of the CD2 pathway by CD3-Ti is intact in the cellular model herein.

Stimulation of IL-2 production through the human CD2 molecule and its variant forms on murine T cells.

We next examined if nuclear activation events including IL-2 induction and subsequent IL-2 secretion could be triggered through the human CD2 molecule in murine CD2 FL cells. To this end, clones were stimulated and IL-2 secretion into supernatants assayed using the IL-2 dependent CTLL-20 cells (Gillis. S. et al., J. Immunol. 120:2077-2081 (1978)). Serial twofold dilutions of culture supernatants from stimulated unactivated murine cells were removed.

$10^5$ cells/well were incubated in 96 well round bottom plates for 24 hours in the presence of either ovalbumin (1 mg/ml final concentration) plus $10^5$ A20-11 B lymphoma cells or anti-$T11_2$ and anti-$T11_3$ (ascites 1:100) or culture medium. Incubation with the non-stimulatory combination of anti-$T11_1$ and anti-$T11_2$ antibodies did not induce any detectable IL-2 production while stimulation with anti-$T11_2$ and anti-$T11_3$ antibodies resulted in clear IL-2 secretion by CD2 FL cells.

Addition of phorbol myristate acetate (PMA, Sigma Chemical) was found to induce a substantial increase in lymphokine production. Thus, 5 ng/ml final concentration of PMA was added to all experimental samples including the media control. Subsequently, supernatants were harvested and titrated in triplicate for their ability to support the growth of 10,000 CTLL-20 cells.

Cultures were pulsed after 24 hours incubation with 1 $\mu$Ci $^{3}$H-thymidine per well and harvested after an additional overnight incubation at 37°C over glass fiber filters on a Mash apparatus. Filters were dried and counted on a beta counter after addition of scintillation fluid. Results are expressed as mean of triplicate determinations of cpm of $^{3}$H-thymidine incorporated. Standard deviations were generally <5-10% and results are representative of five independent experiments. As shown in Figure 9, supernatants from CD2 FL or 3DO54.8 cells stimulated with ovalbumin in the presence of the H-2(I-$A^{d}$) expressing A20-11 B lymphoma are able to induce proliferation of the IL-2 dependent CTLL-20 cells in a comparable way. By contrast, the combination of anti-$Tll_2$ and anti-$Tll_3$ antibodies is effective in inducing IL-2 production by CD2 FL cells but not the parental line 3DO54.8.

IL-2 production upon antigen (ovalbumin) or anti-$Tll_2$ and anti-$Tll_3$ stimulation was determined using the above methods. IL-2 production was obtained by running culture supernatants in parallel to a titration curve of recombinant IL-2 (Biogen Labs,

Cambridge, MA). The limit of detection for IL-2 was <4 U/ml. As shown in Figures 10a and 10b, M represents the murine T cell line CD2 M271-2. Analysis of IL-2 production by two independent clones of the CD2 M278-9 provided evidence that such cells could produce significant amounts of IL-2 upon CD2 triggering (approximately 50% of the amount secreted upon antigen stimulation). Furthermore, all of the cell lines tested including CD2 FL, 3DO54-8 and CD2ΔC series produce a high amount of IL-2 (ranging from 30-200 U/ml corresponding to a clonal variation repeatedly observed) when stimulated with ovalbumin in the I-$A^d$ context.

This result demonstrates the integrity of the IL-2 synthetic pathway in each clone. Perhaps more importantly, after stimulation with anti-CD2 antibodies, the clones CD2 FL, CD2ΔC98 and CD2ΔC77 produce a comparable level of IL-2 while transfectants CD2ΔC43 and CD2ΔC18, like the untransfected 3DO54.8, are not triggered through CD2. Analysis of five other independent clones expressing the ΔC43 CD2 molecule clearly established that these cells are not triggered through human CD2 either to secrete detectable levels of IL-2 or to elevate $[Ca^{2+}]_i$. Taken together, these data show that a full length human CD2 molecule, as well as a CD2 molecule lacking 19 or 40 carboxy-terminal amino acids from the cytoplasmic domain, is able to activate T lymphocytes after appropriate perturbation of the CD2 extracellular segment.

Interestingly, the CD2ΔC77 clones express human CD2 molecules lacking residues 289 to 316. The latter c rresponds to the segment most c nserved among human

and murine molecules with 24 out of 27 residues being identical (Figures 2 and 3). Presumably, these conserved residues function in another facet of CD2 biology unrelated to IL-2 induction and/or secretion. In contrast, the ΔC43 truncated molecules, as well as shorter truncations are non-functional with respect to stimulating a rise in $[Ca^{+2}]_i$ and IL-2 production.

These data provide unequivocal evidence that the CD2 cytoplasmic domain is involved in signal transduction and that one essential sequence of the cytoplasmic domain necessary for CD2-mediated activation is located between amino acids 253 and 287. This region contains four histidines at amino acid positions 264, 271, 278 and 282 and includes two tandemly repeated segments (PPPGHR, amino acids 260-265 and 274-279) (see Figures 2 and 3). These histidine residues could represent a binding site for an ion, cyclic nucleotide or other small regulatory molecule.

Evaluation of the role of the histidine residues of the human CD2 cytoplasmic domain

To evaluate the role of the histidine residues, substitution mutants of CD2 were produced by site-directed mutagenesis. Two different categories of mutants with a stable CD2 surface expression were obtained: CD2 M172-2 and CD2 M278-9 in which the positively charged histidine and arginine residues at position 271 and 272 or 278 and 279 were replaced by negatively charged aspartic acid and glutamic acid, respectively (Figure 3). Functional characterization of these two mutants shows that IL-2 production can be

induced by anti-$T11_2$ and anti-$T11_3$ antibodies to a level comparable to that of the CD2 clones (Figure 10). Thus, a putative "cage", requiring four histidine residues, is not necessary for this CD2 activation. Furthermore, since the mutations at position 278-279 alters the structure of the second repeat without affecting T cell activation, the more carboxy-terminal repeat is not required.

Deposit

DOL vectors containing CD2FL, CD2ΔC77 or CD2ΔC98 (CD2 truncated cDNA) have been deposited in the American Type Culture Collection, Rockville, Maryland, on May 2, 1989, under the terms of the Budapest Treaty. Accession numbers 40599, 40598 and 40600, respectively, have been assigned to them.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

International Applicati n No: PCT/ /

## MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 25, line 10 of the description [1]

A. IDENTIFICATION OF DEPOSIT [2]

Further deposits are identified on an additional sheet [X] [3]

Name of depositary institution [4]

American Type Culture Collection

Address of depositary institution (including postal code and country) [4]

12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

| Date of deposit [5] | Accession Number [6] |
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| 02 MAY 1989 (02.05.89) | 40599 |

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(January 1985)

International Application No: PCT/ /

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**A. IDENTIFICATION OF DEPOSIT** [2]

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American Type Culture Collection

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## CLAIMS

1. A soluble peptide having an amino acid sequence that corresponds to the cytoplasmic domain of human CD2 glycoprotein, or a portion thereof, which is necessary for transduction of a CD2-mediated T lymphocyte activation signal into a cell.

2. A soluble peptide of Claim 1 having an amino acid sequence which corresponds to the amino-terminal portion of the cytoplasmic domain of human CD2 glycoprotein or fragment thereof which is necessary for transduction of a CD2-mediated T lymphocyte activation signal into a cell.